ELYRA – 3D PALM Experimental PSF Multichannel Alignment

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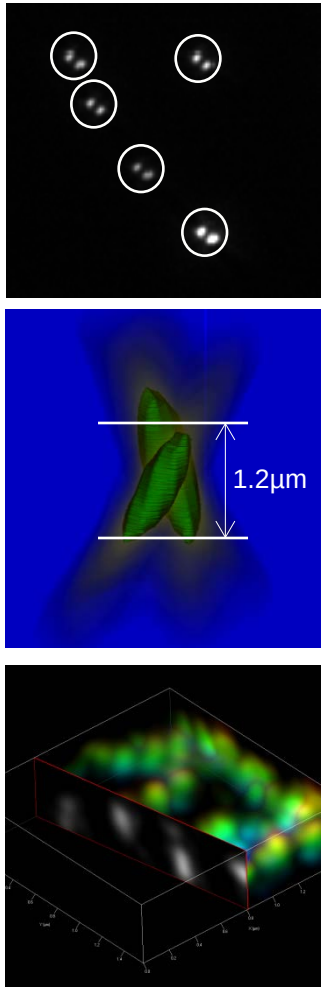
October 2, 2015

Outline

Workflow for 3D PALM on ELYRA P.1 or PS.1



1. Acquire PSF
2. Experimental PSF

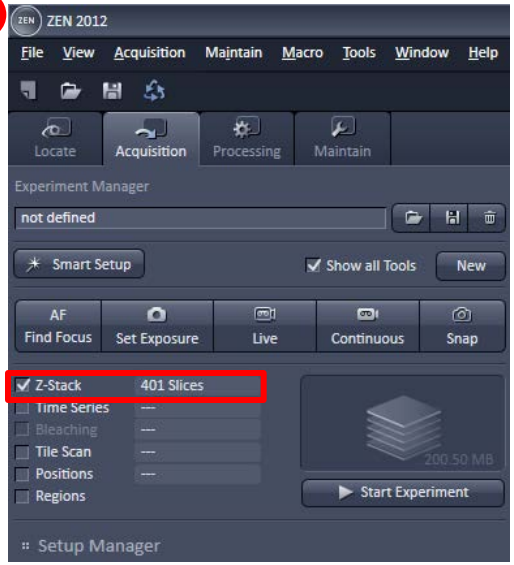
Automatic

3. Create Localization LUT
4. Apply to Images

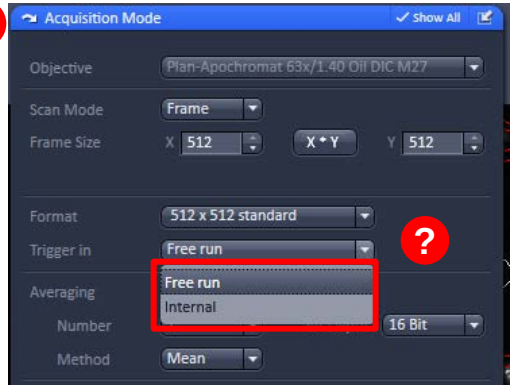
1. Acquire PSF

Using (100 nm tetraspec) beads, 3D slider in and 4 µm range

1



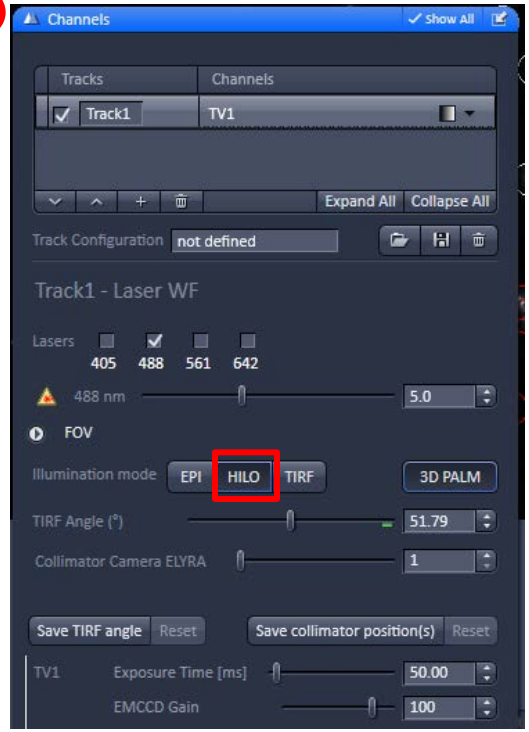
2



internal: recommended
free run: works

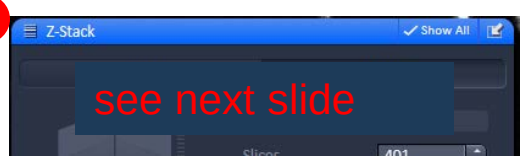
3

4



HILO: recommended
time / gain typical: 50 ms / 100 gain

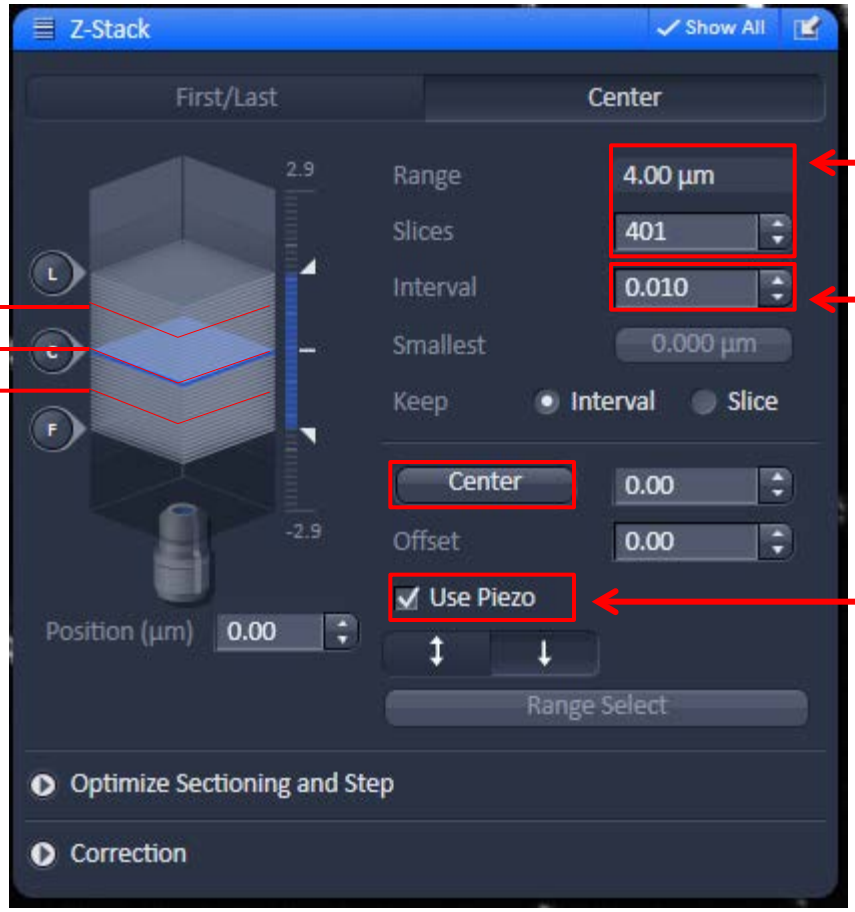
1



z-stack settings: see next slide

1. Acquire PSF

Z-stack definition



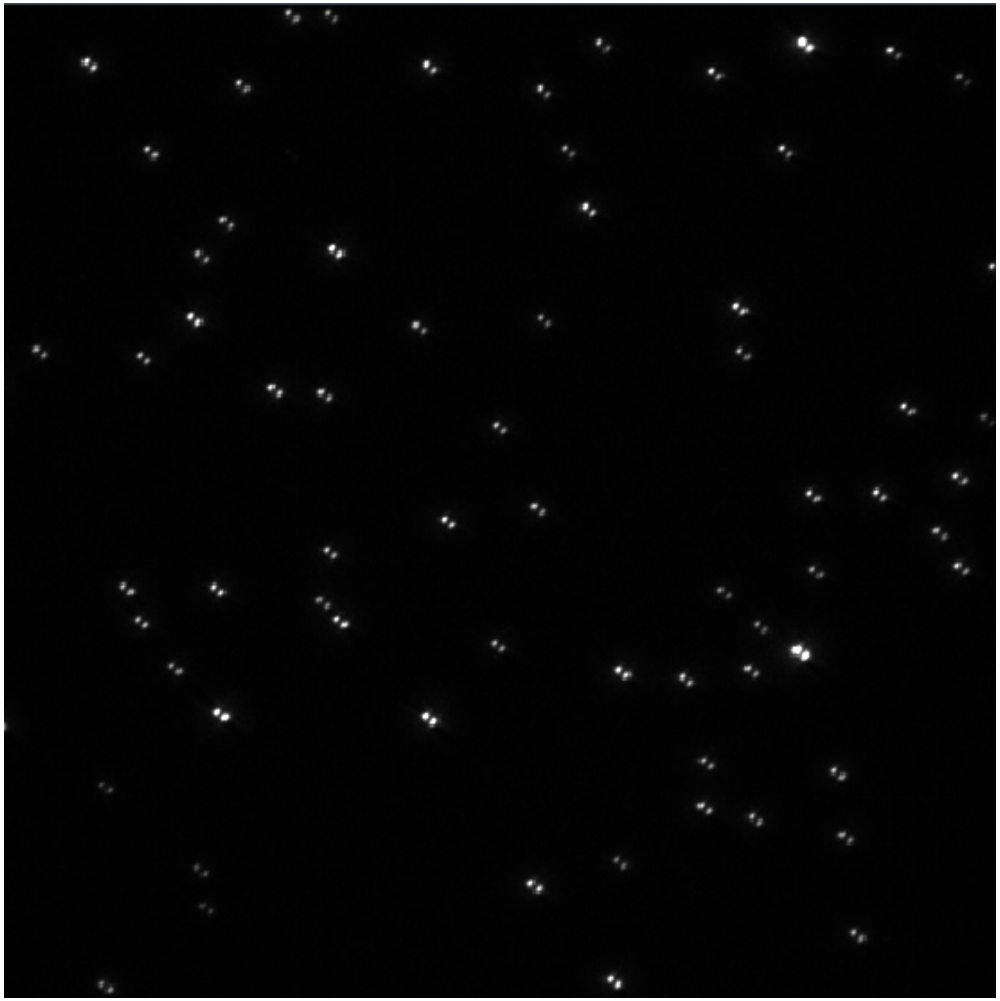
Use 4 µm range
to avoid artefacts

10 nm step size
for good oversampling

Use piezo
make sure this is active

1. A typically suitable z-stack

Sparse beads, well separated lobes, in dynamic range



Rules of Thumb:

(1) approx. 10 - 80 beads in FOV

If fewer: poor statistics
If too crowded: too many beads discarded

Remedy: Look for region in
sample with appropriate density

(2) Exploit full dynamic range

Avoid too bright bead agglomerates in FOV

(3) Lobes well separated

Check oil (bubbles)
Check 3D slider (Maintain tab)
Check collimator position
Vary HILO angle
Use other objective

Outline

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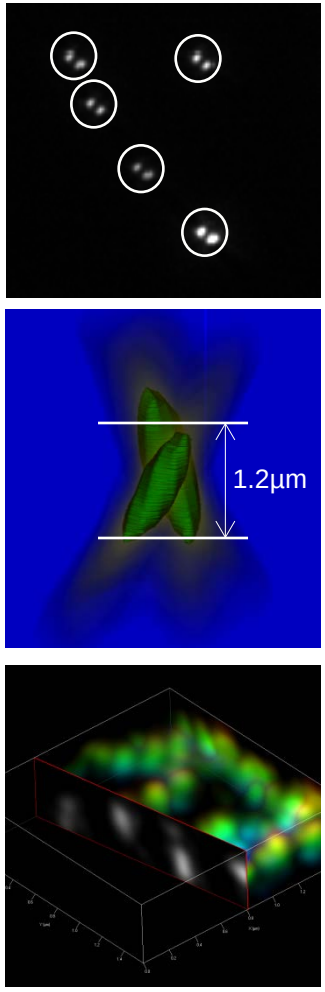

1. Acquire PSF ✓
2. Experimental PSF
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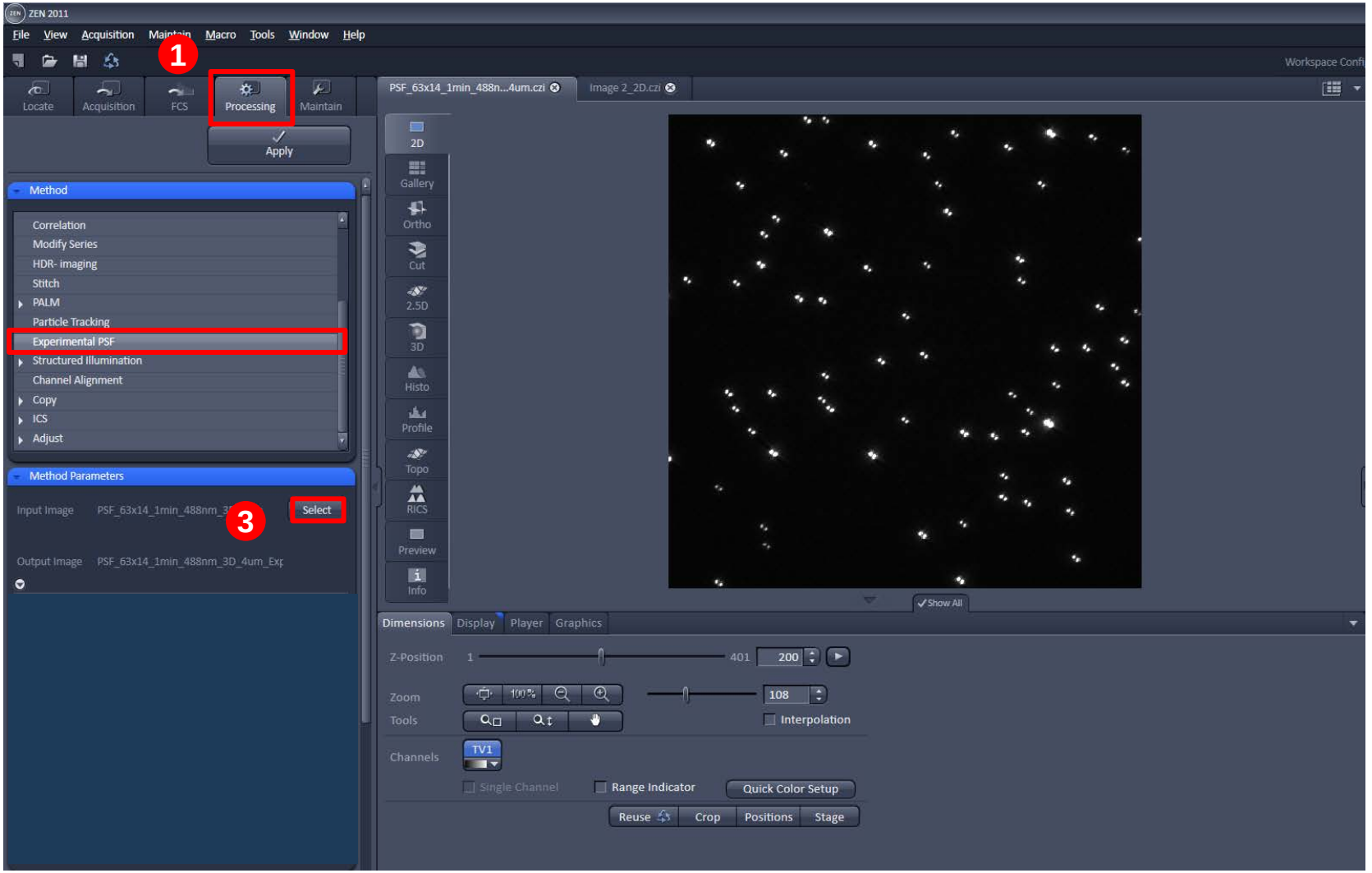
5. Apply to Images

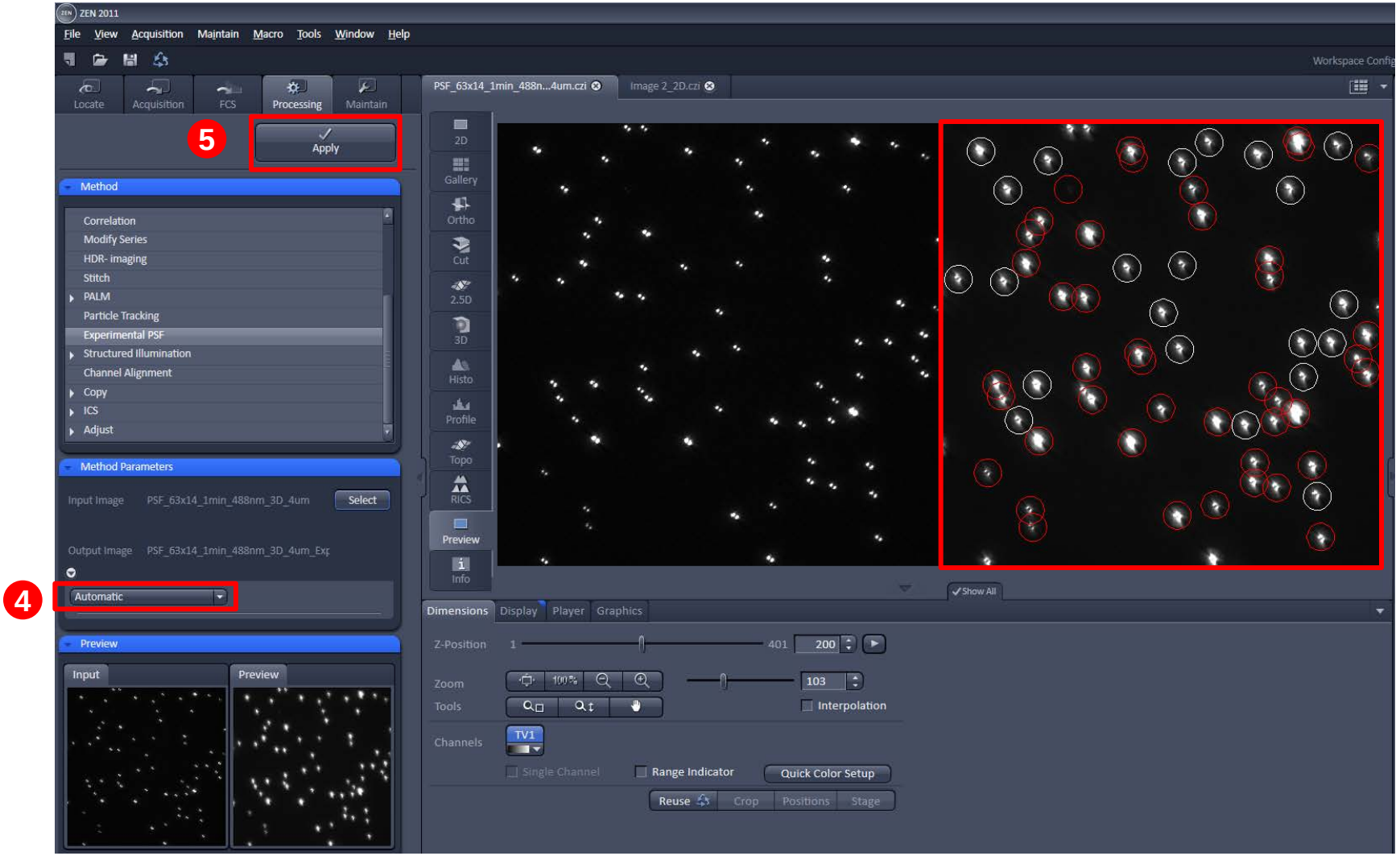
2. Build Experimental PSF

Processing Tab, Experimental PSF, Select



2. Build Experimental PSF

Automatically recognizes 3D-PALM situation

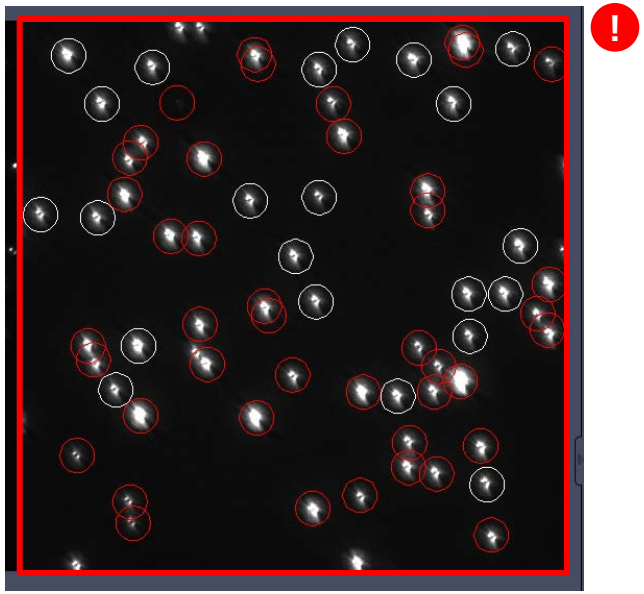


2. Build Experimental PSF

Bead selection display in preview

ZEN will only use well-behaved patterns:

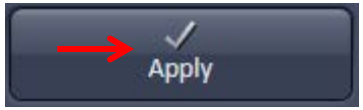
White circles show which patterns are used
Red circles show the discarded patterns



Discarding may be due to:

(a) Overlap between patterns
(b) SNR too low
(c) Pattern too close to edge of image

Possibilities

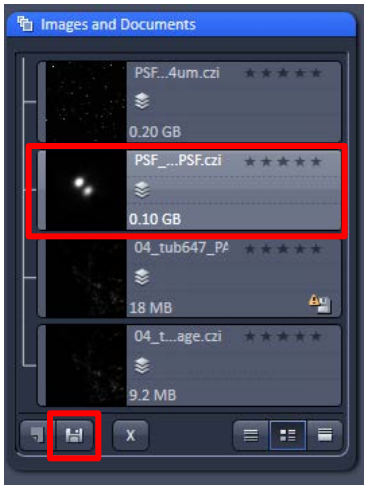


(1) Accept auto settings as are and go (Apply)
(2) Inspect selection closer (by moving slider)
(3) Refine / Modify selection criteria (manual)

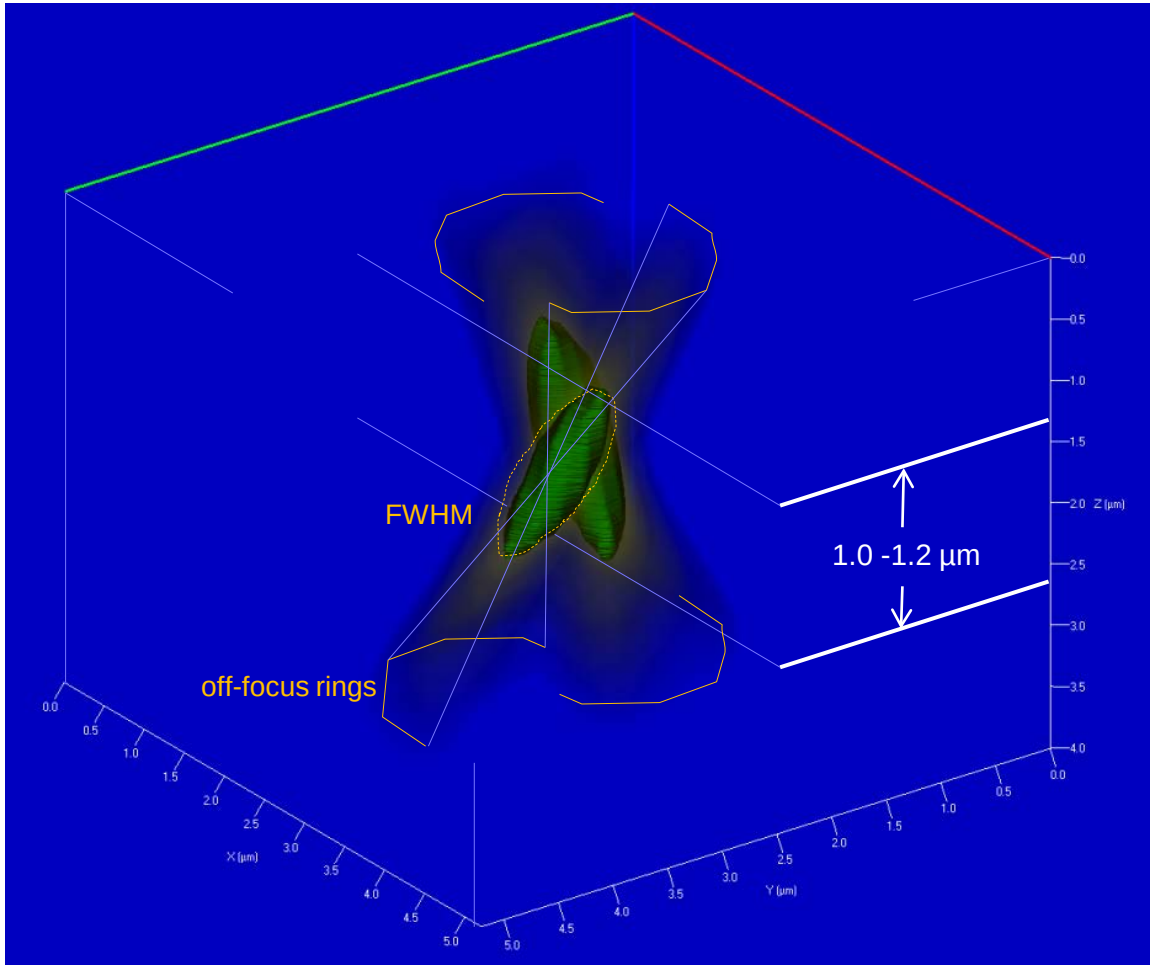
approx. 60 total
approx. 20 selected

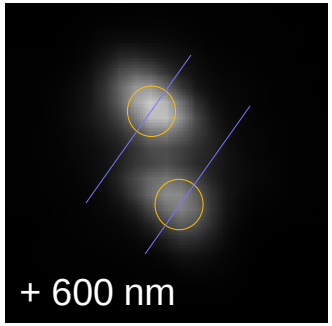
2. Build Experimental PSF

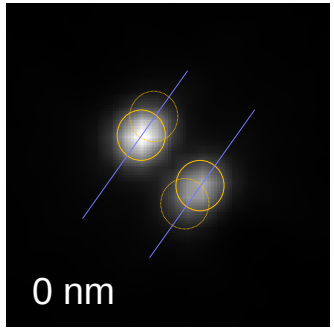
PSF extends over several microns

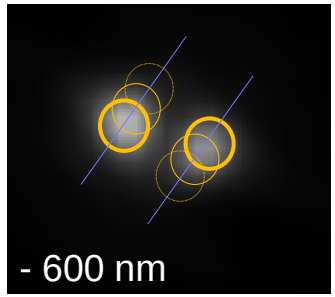


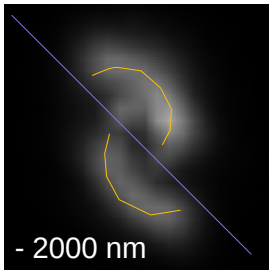
SAVE!











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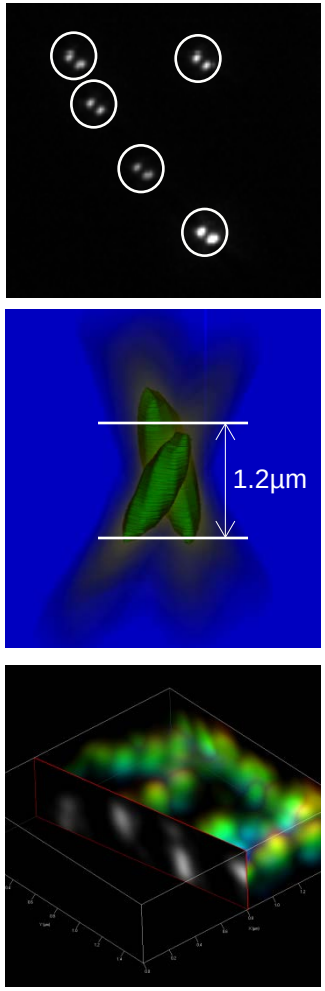
Workflow for 3D PALM on ELYRA P.1 or PS.1

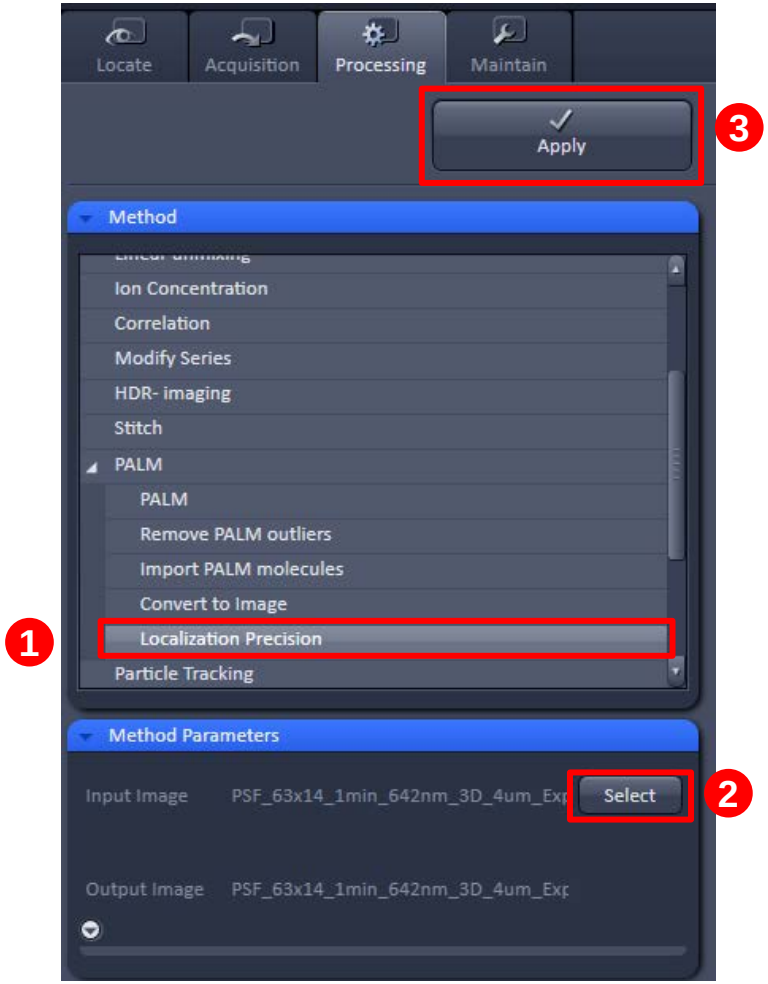


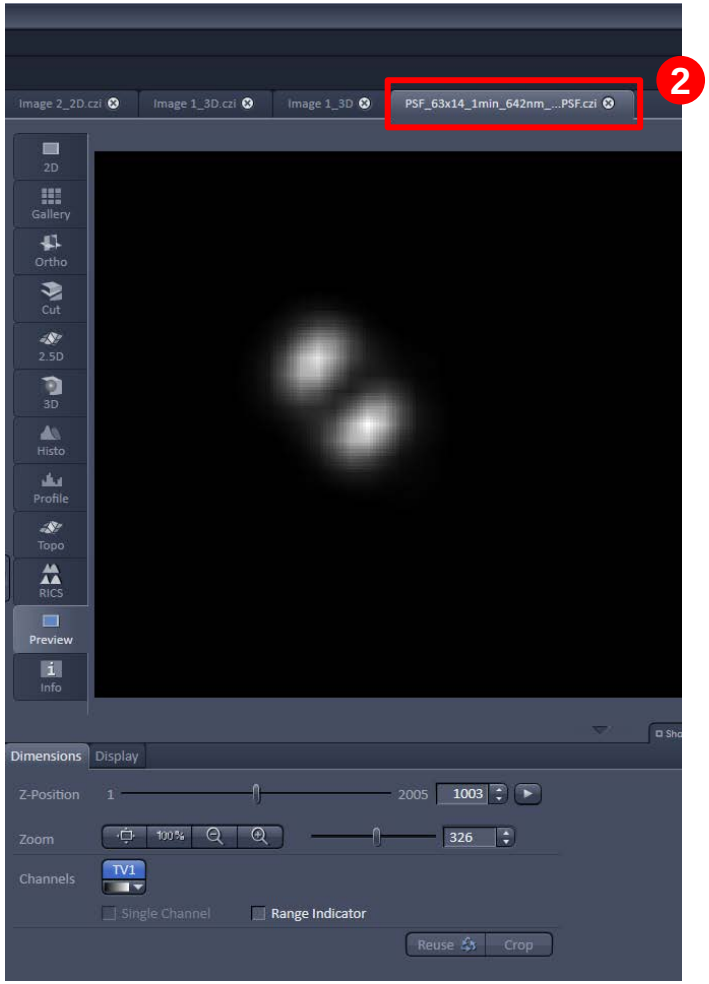
1. Acquire PSF ✓
2. Experimental PSF
3. Create Localization LUT
4. Apply to Images

✓ Automatic

3. Create Localization Precision LUT

Localization Precision, Select Experimental PSF File, Apply.

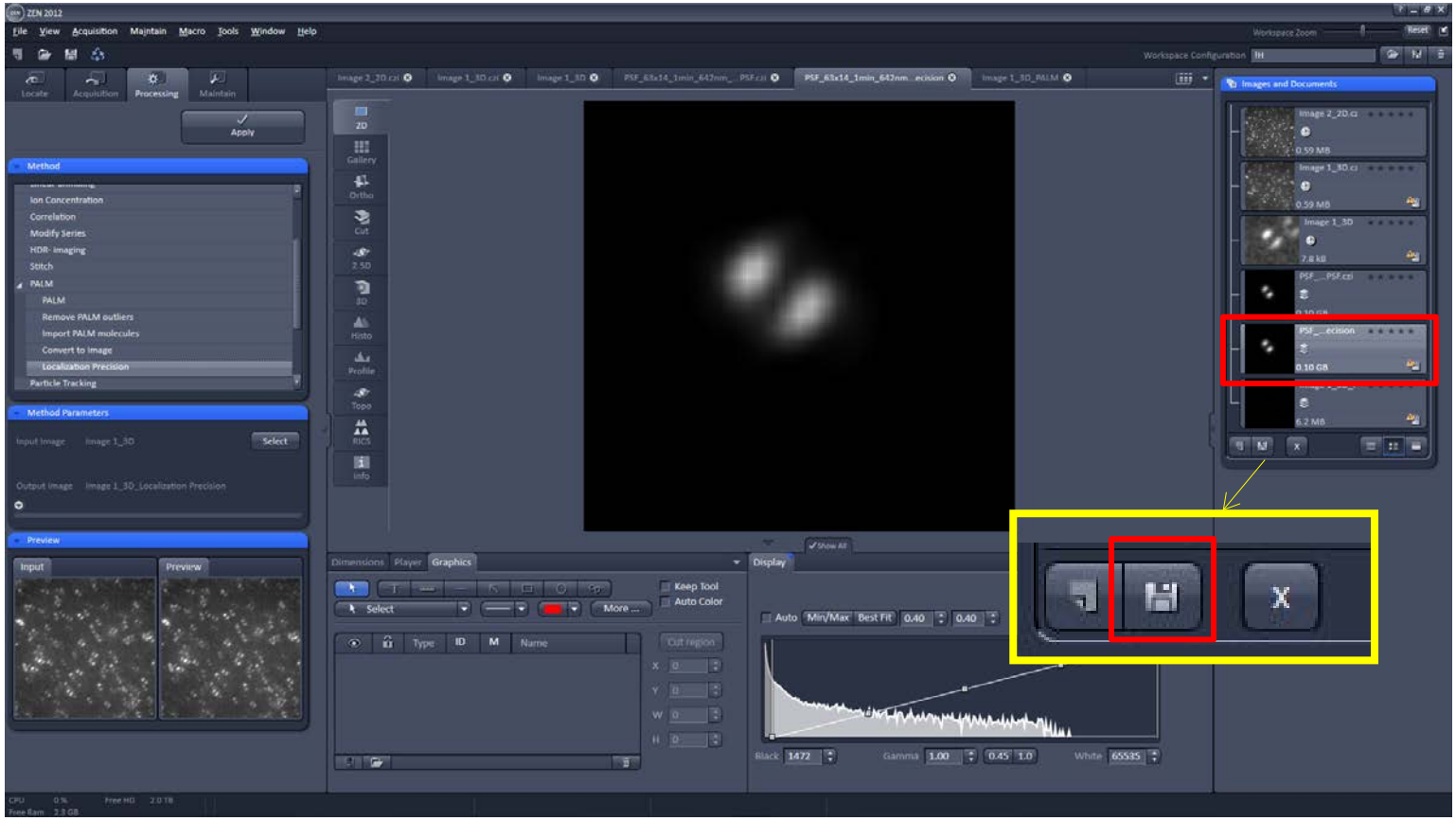




! Takes a few minutes!

3. Create Localization Precision LUT

After it is done: Just save.



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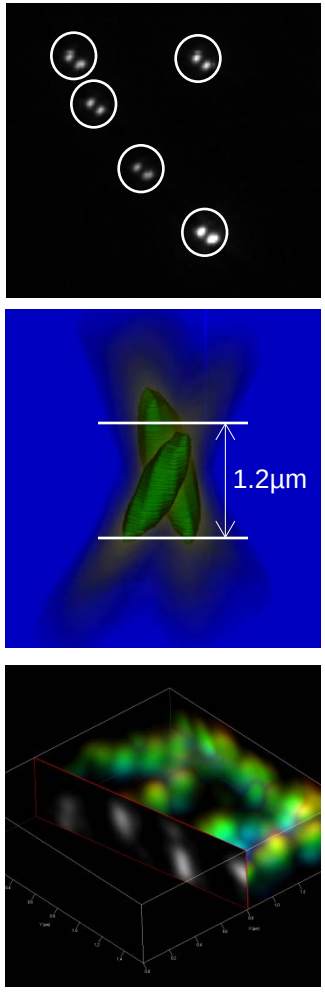
Workflow for 3D PALM on ELYRA P.1 or PS.1



1. Acquire PSF ✓
2. Experimental PSF
3. Automatic ✓
4. Create Localization LUT ✓
5. Apply to Images

4. Apply PSF File to images

Load sequence, assign proper Precision LUT and go.

(1) Processing -> PALM

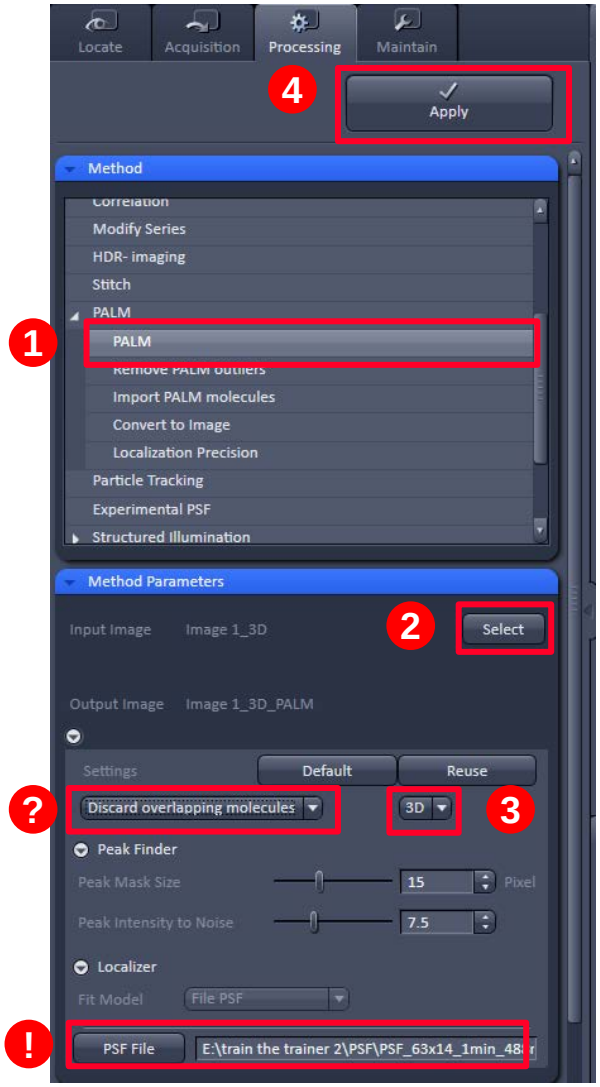
(2) select image

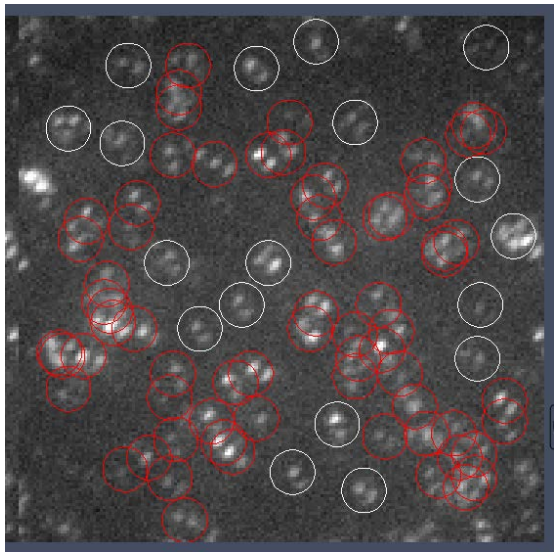
(!) select LUT: this is your experimental PSF you just created

(?) choose:
(a) account for overlap (slow, more patterns)
(b) discard overlap (fast, lots of discarding)
(c) ignore overlap (more errors)

(3) 3D (automatically selected)

(4) Apply





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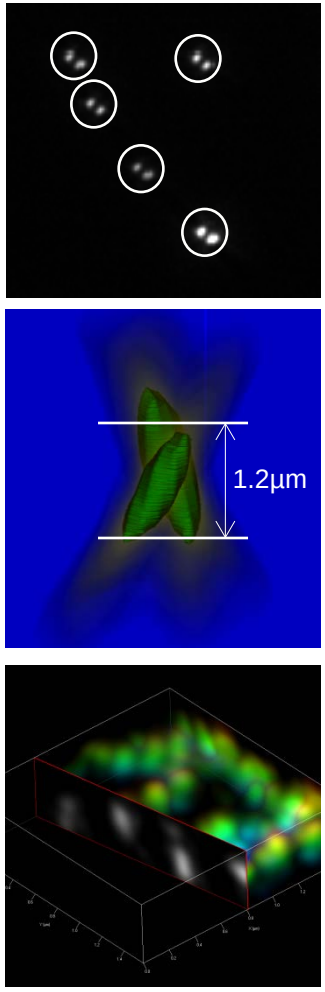


1. Acquire PSF ✓
2. Experimental PSF
3. Automatic ✓
4. Create Localization LUT ✓
5. Apply to Images ✓

3D PALM Multicolor Channel Alignment

Procedure:

Requirement: There must be fiducials present in the images of both channels.

1) After processing the raw data, if both channels were acquired together separate each color as its own PALM file.
   a. Perform fiducial drift correction on each channel image
   b. Write down the x, y, z positions of the common fiducials between each color
   c. Render the images with the desired lateral and axial resolution. Be sure to check that both colors have exactly the same pixel size and z-plane thickness.
   d. Convert each channel to an image

2) Combine the two “convert to image” files into one image using the Channel Alignment function
   a. Select the two colors into “input 1” and “input 2”, check “input 2” box, uncheck “Fit” and apply.
   b. Note: the data should be simply combined and not shifted/corrected.
   c. Note: The file size of the combined image must be less than 4 GB.

3D PALM Multicolor Channel Alignment

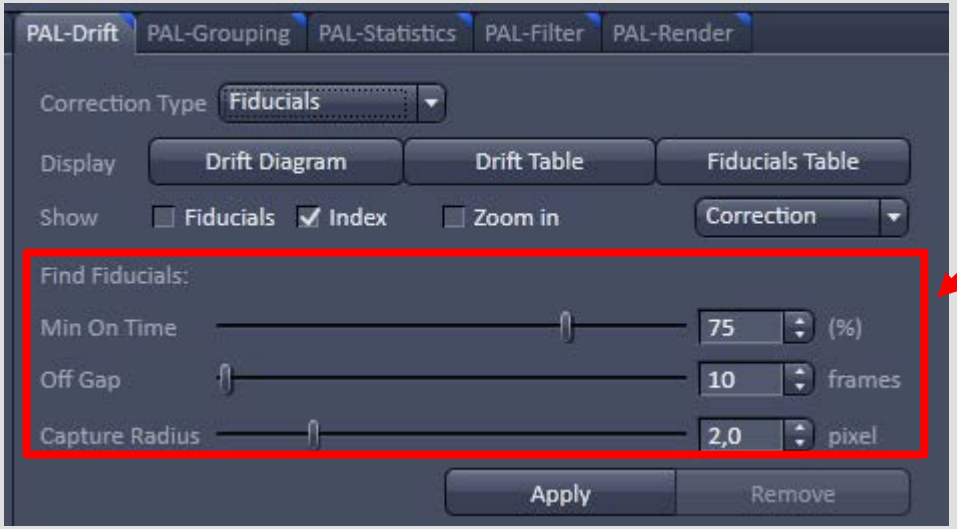
3) Use the Channel Alignment function again on the resulting image from step 2.
   a. Select the result from step 2 above into “input 1”
   b. Uncheck “input2”, uncheck “fit”
   c. Based on the x,y,z positions of the corrected fiducials you wrote in step **1b** above, calculate the average pixel shifts required for the channel alignment function:
      i. Fiducial_A = (Xa, Ya, Za), and Fiducial_B = (Xb, Yb, Zb)
      ii. Shift coefficient X = Xa-Xb / pixel size
      iii. Shift coefficient Y = Ya-Yb / pixel size
      iv. Shift coefficient Z = Za-Zb / zSliceThickness
   d. Using only “LATERAL” alignment, manually enter the XYZ coefficients above into the channel alignment function.
      i. Make sure the channels are properly matched (ID = 0 is matched to first channel, ID = 1 matched to second channel)
   e. Hit Apply

Note 1: Consider the sign of the coefficients to make sure they are not flipped.
Note 2: The quality of the color fits depend critically on the quality of the fiducial drift correction. If you have poor fiducials or very few (2 or less), then the alignment may not be accurate.

3D PALM Multicolor Channel Alignment

1a. Drift Correct using Fiducials

Automatically detect fiducials:



Definition to auto-find fiducials.
Fiducials can also be hand-picked

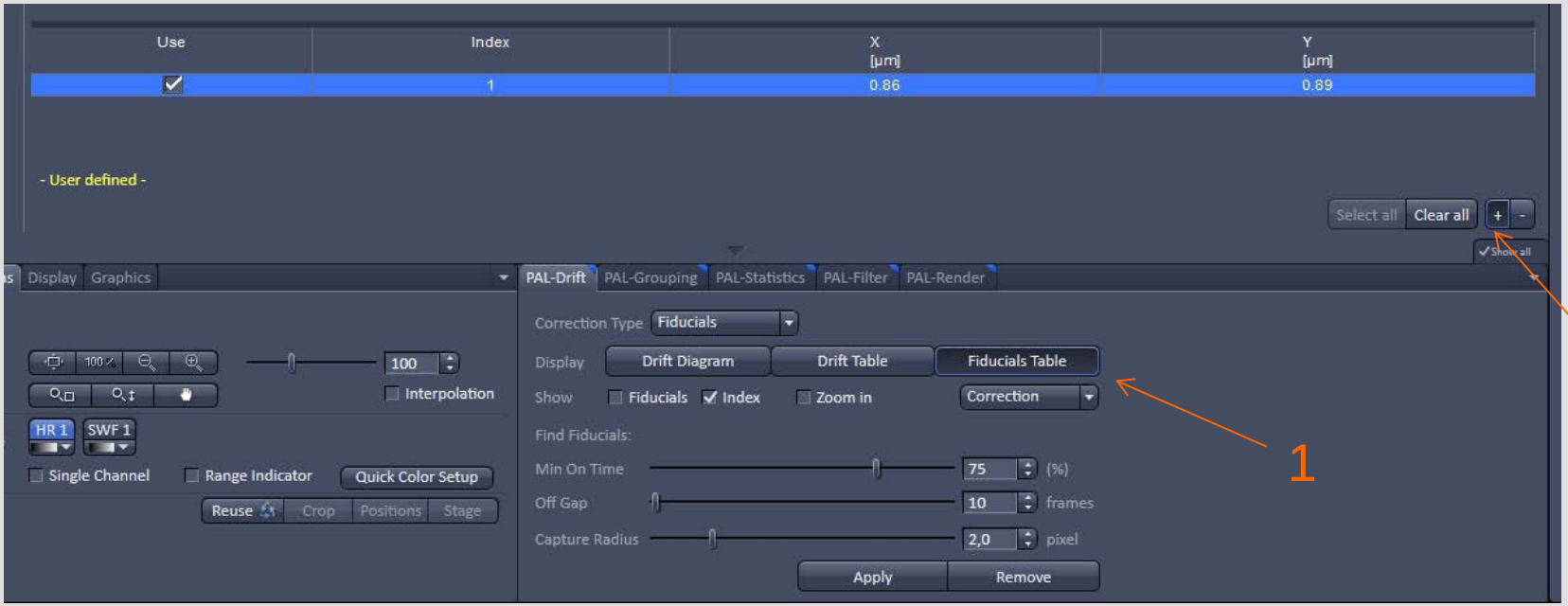
Hand-picking fiducials:
see next slide

Camera pixel corresponds to 100nm
(e.g. Capture Radius 2,0 = 200 nm)

3D PALM Multicolor Channel Alignment

1a. Drift Correct using Fiducials

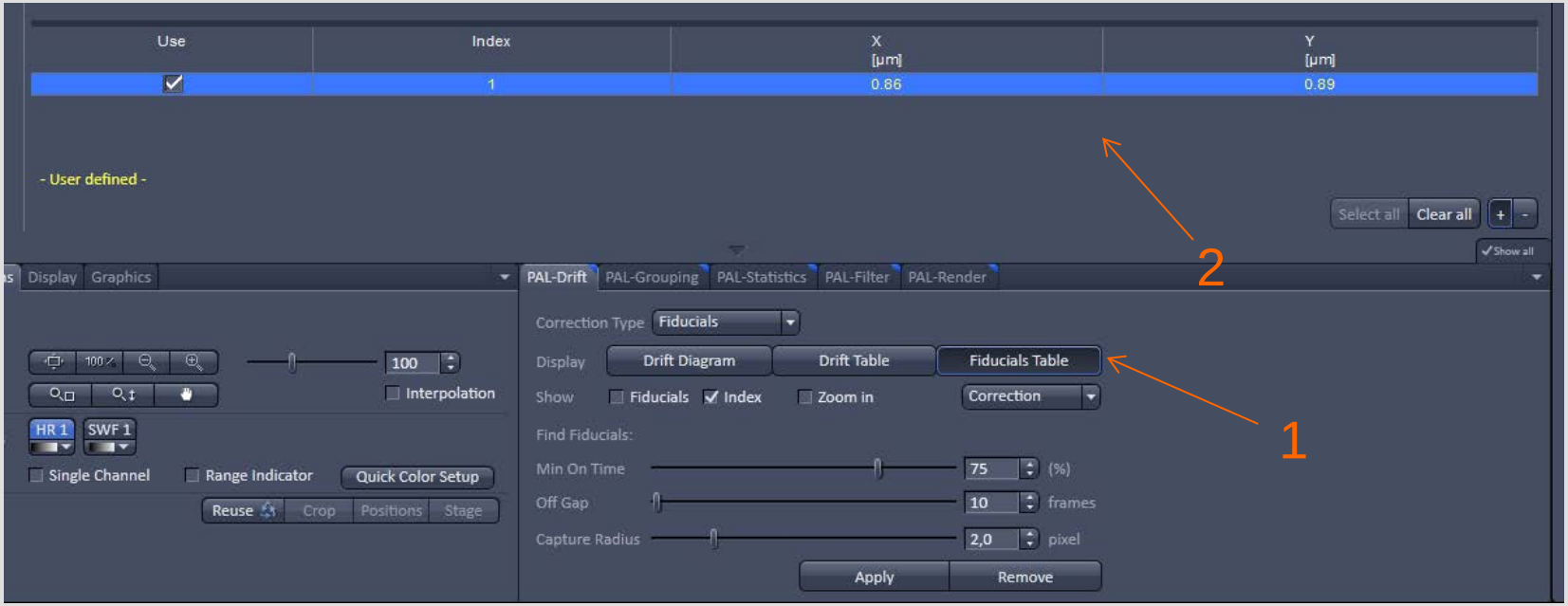
Hand select fiducials:



1. Select Fiducials Table
2. Click on (+) then hover over in image and click to select fiducial

3D PALM Multicolor Channel Alignment

1b. Write down x, y, z, positions of fiducials



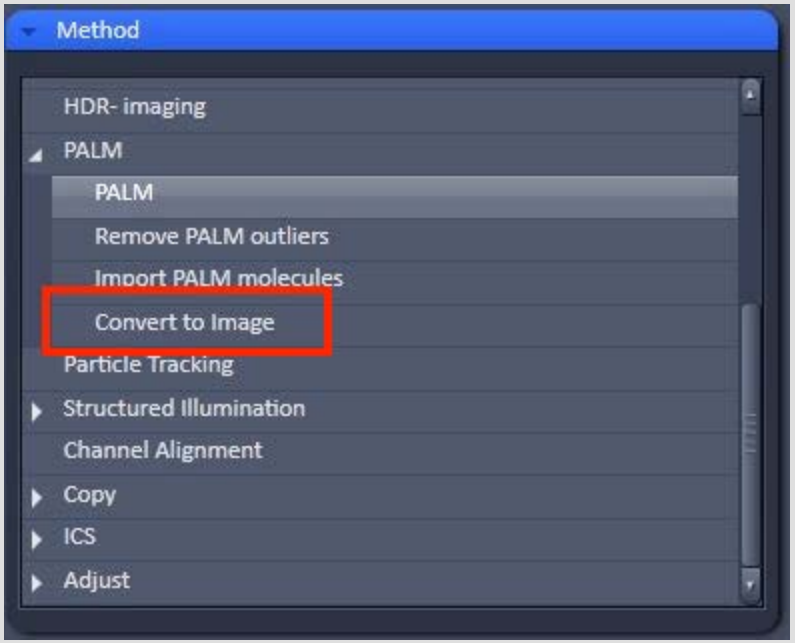
1. Select Fiducials Table
2. Write down the x, y, z positions of the common fiducials between each color

3D PALM Multicolor Channel Alignment

1c-d. Render and convert to images

c. Render the images with the desired lateral and axial resolution. Be sure to check that both colors have exactly the same pixel size and z-plane thickness.

d. Convert each channel to an image

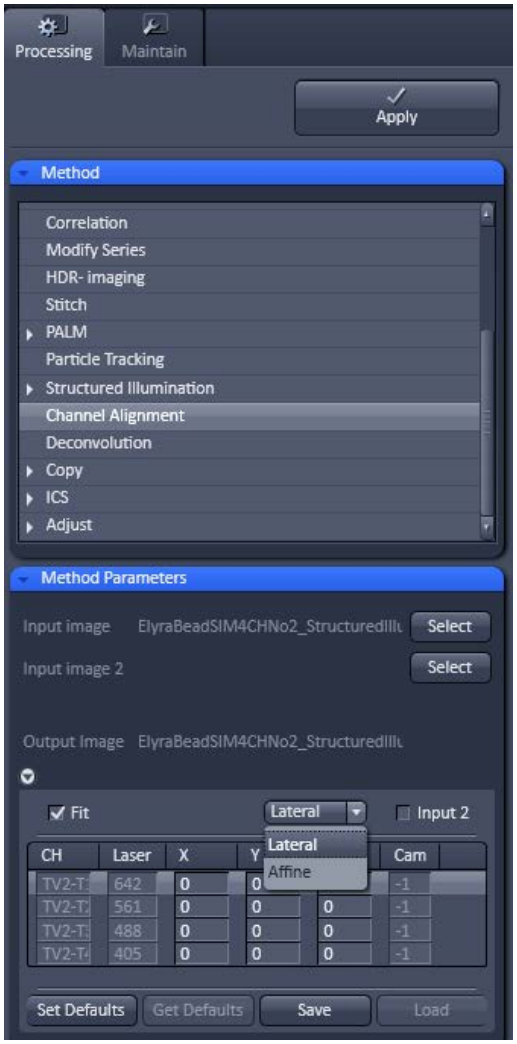


3D PALM Multicolor Channel Alignment

2. Combine the two “convert to image” files

2) Combine the two “convert to image” files into one image using the Channel Alignment function

a. Select the two colors into “input 1” and “input 2”, check “input 2” box, uncheck “Fit” and apply.
b. Note: the data should be simply combined and not shifted/corrected.
c. Note: The file size of the combined image must be less than 4 GB. If not, reduce the number of pixels or size of image.



Uncheck “Fit”

Select Ch 1 image

Select Ch 2 image

Check “Input 2”

3D PALM Multicolor Channel Alignment

3. Calculate shifts and align channels

3) Use the Channel Alignment function again on the resulting image from step 2.

a. Select the result from step 2 above into “input 1”
b. Uncheck “input2”, uncheck “fit”
c. Based on the x,y,z positions of the corrected fiducials you wrote in step **1b** above, calculate the average pixel shifts required for the channel alignment function:
   i. Fiducial_A = (Xa, Ya, Za), and Fiducial_B = (Xb, Yb, Zb)
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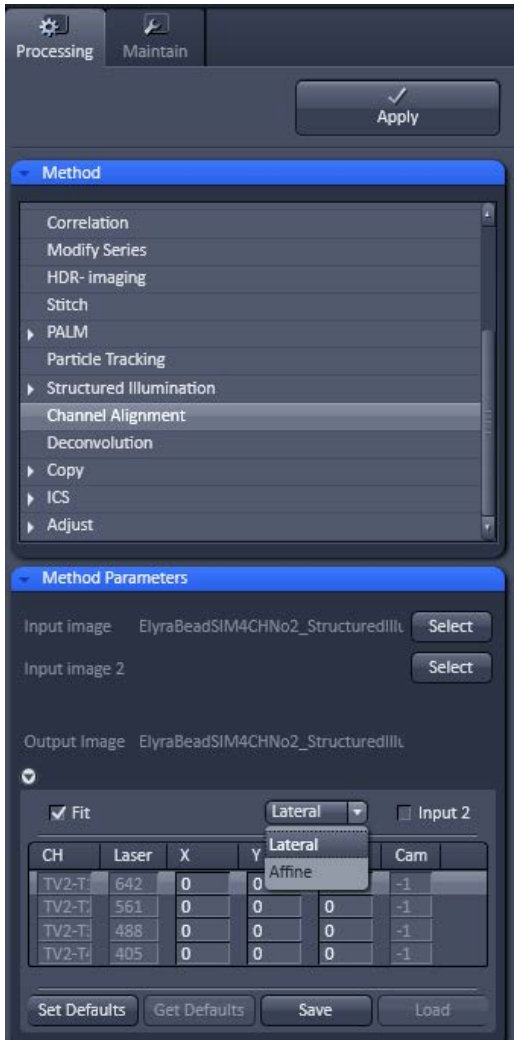


Uncheck “Fit”

Select combined image with “Input 1”

Uncheck “Input 2”

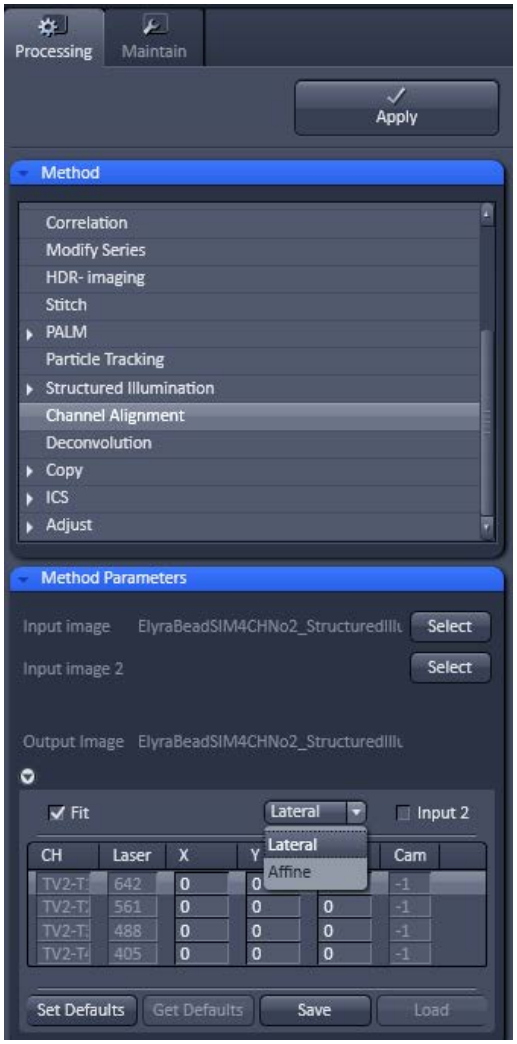
Continued on next slide…

3D PALM Multicolor Channel Alignment

a. Using only “LATERAL” alignment, manually enter the XYZ coefficients into the channel alignment function.
   i. Make sure the channels are properly matched (ID = 0 is matched to first channel, ID = 1 matched to second channel)
b. Hit Apply

Note 1: Consider the sign of the coefficients to make sure they are not flipped.

Note 2: The quality of the color fits depend critically on the quality of the fiducial drift correction. If you have poor fiducials or very few (2 or less), then the alignment may not be accurate.



Choose “Lateral”

Fill in desired x, y, z shifts

We make it visible.